

University of Groningen

Directed evolution of an enantioselective *Bacillus subtilis* lipase

Funke, SA; Eipper, A; Reetz, MT; Otte, N; Thiel, W; Van Pouderoyen, G; Dijkstra, BW; Jaeger, KE; Eggert, T; Funke, Susanne Aileen

Published in:
Biocatalysis and Biotransformation

DOI:
[10.1080/1024242031000110847](https://doi.org/10.1080/1024242031000110847)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Funke, SA., Eipper, A., Reetz, MT., Otte, N., Thiel, W., Van Pouderoyen, G., Dijkstra, BW., Jaeger, KE., Eggert, T., Funke, S. A., Reetz, M. T., Dijkstra, B. W., & Jaeger, K-E. (2003). Directed evolution of an enantioselective *Bacillus subtilis* lipase. *Biocatalysis and Biotransformation*, 21(2), 67-73.
<https://doi.org/10.1080/1024242031000110847>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Directed Evolution of an Enantioselective *Bacillus subtilis* Lipase

SUSANNE AILEEN FUNKE^a, ANDREAS EIPPER^{§b}, MANFRED T. REETZ^b, NIKOLAJ OTTE^c,
WALTER THIEL^c, GERTIE VAN POUDEROYEN^d, BAUKE W. DIJKSTRA^d, KARL-ERICH JAEGER^a and
THORSTEN EGGERT^{a*}

^a Institut für Molekulare Enzymtechnologie, Heinrich-Heine-Universität Düsseldorf, D-52426 Jülich, Germany; ^b Synthetische Organische Chemie, Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, D-45470 Mülheim an der Ruhr, Germany; ^c Theoretische Chemie, Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, D-45470 Mülheim an der Ruhr, Germany; ^d Laboratory of Biophysical Chemistry, Rijksuniversiteit Groningen, Nijenborgh 4, NL-9747 AG Groningen, The Netherlands

Chiral compounds are of steadily increasing importance to the chemical industry, in particular for the production of pharmaceuticals. Where do these compounds come from? Apart from natural resources, two synthetic strategies are available: asymmetric chemical catalysis using transition metal catalysts and biocatalysis using enzymes. In the latter case, screening programs have identified a number of enzymes. However, their enantioselectivity is often not high enough for a desired reaction. This problem can be solved by applying directed evolution to create enantioselective enzymes as shown here for a lipase from *Bacillus subtilis*. The reaction studied was the asymmetric hydrolysis of *meso*-1,4-diacetoxy-2-cyclopentene with the formation of chiral alcohols which were detected by electrospray ionization mass spectrometry. Iterative cycles of random mutagenesis and screening allowed the identification of several variants with improved enantioselectivities. In parallel, we have started to use X-ray structural data to simulate the *Bacillus subtilis* lipase A-catalyzed substrate hydrolysis by using quantum mechanical and molecular mechanical calculations. This combined approach should finally enable us to devise more efficient strategies for the directed evolution of enantioselective enzymes.

Keywords: Directed evolution; Enantioselectivity; Lipase; Esterase; *Bacillus subtilis*; Saturation mutagenesis

INTRODUCTION

The world market for enantiomerically pure compounds is increasing, with the worldwide sales

volume for single enantiomers reaching \$6.63 billion in 2001 (Stinson, 2001). The necessity to use enantiomerically pure compounds as active ingredients in pharmaceuticals will result in a further sales volume increase for enantiomerically pure building blocks to reach \$15.1 billion in 2005 (Rouhi, 2002). When looking at the chiral drugs market, the numbers are even more impressive: in 2001, single enantiomer drug sales reached \$147 billion, which corresponds to a market share of 36% for the worldwide pharmaceutical products (Rouhi, 2002).

Single enantiomers become available either by isolation from natural sources (the so-called chiral pool) or by asymmetric chemical catalysis using transition metal catalysts. In addition, biocatalysis has evolved as an attractive alternative, with lipases being the most widely used enzymes which work in aqueous as well as organic solvents and catalyze a large variety of different reactions with a high substrate specificity and stereoselectivity (Jaeger and Eggert, 2002; Reetz, 2002). However, biocatalysts generally suffer from the disadvantage that for a given synthetic transformation of interest, $A \rightarrow B$, enantioselectivity may well be poor. This problem can be solved by improving the enantioselectivity of enzymes using directed evolution (for reviews see Bornscheuer and Pohl, 2001; Jaeger *et al.*, 2001; Reetz and Jaeger, 2002). This strategy includes (i) the generation of mutant enzyme genes using different mutagenesis methods like error-prone polymerase chain reaction, saturation mutagenesis and *in vitro* recombination (Stemmer, 1994a,b; Zhao *et al.*, 1998; Shao *et al.*, 2000; Lutz *et al.*, 2001), (ii) followed by gene expression to generate the corresponding en-

[§] present address: BASF AG, Carl-Bosch-Straße 38, 67056 Ludwigshafen, Germany

* Corresponding author. Tel.: +49-2461-61-2939. Fax: +49-2461-61-2490. E-mail: t.eggert@fz-juelich.de

zyme variants (Rosenau and Jaeger, 2003) and (iii) high-throughput screening for enantioselectivity (Reetz, 2001).

One general problem in evolving (enantioselective) enzymes concerns the choice of an appropriate mutagenesis method to be used for generating a first generation library. In most cases, epPCR is used followed by recombinative methods and/or saturation mutagenesis. However, single base substitutions introduced by epPCR result in a limited number of amino acid exchanges introduced into the enzyme. In other words, only a small part of the total sequence space is susceptible to mutagenesis and alternative methods must be applied to generate a first generation library of high diversity (Eggert and Jaeger, 2003).

We have used directed evolution to improve the enantioselectivity of *Bacillus subtilis* lipase A (BSLA) in the asymmetric hydrolysis of *meso*-1,4-diacetoxy-2-cyclopentene with the formation of chiral alcohols (Fig. 1). Different directed evolution strategies were compared to evaluate their potential for the efficient creation of enantioselective BSLA variants.

MATERIALS AND METHODS

Heterologous Expression of BSLA in *Escherichia coli*

The lipase gene *lipA* lacking a 93 bp fragment at the 5'-end which encodes its signal sequence was amplified by standard PCR using the upstream 30 bp primer BSLA1 5'-ATATGATATCGCTGAACA-CAATCCAGTCGT-3', the downstream 29 bp-primer BSLA2 5'-TATAGAGCTCTCATTAATTCG-TATTCTGG-3' and genomic DNA from *B. subtilis* 168 (obtained from the *Bacillus* Genetic Stock Center, Ohio, USA) as a template. Unique *EcoRV* and *SacI* restriction sites were introduced and used to clone the resulting 557 bp PCR product into the corresponding restriction sites of the *Escherichia coli* expression plasmid pET22b (Novagen, Madison, USA) giving pET22lipA.

Standard PCR Conditions

Amplification of DNA fragments was performed in a 50 μ l reaction mix containing 1 ng plasmid- or 10 ng genomic-DNA as the template, 25 pmol of each primer, 0.2 mM dNTP's, 2.5 U *Taq*- (Eurogentec, Seraing, Belgium) or *Pfu*- (Stratagene, Heidelberg, Germany) polymerase. Buffers containing $MgCl_2$ or $MgSO_4$ were used as recommended by the manufacturers. Conditions for PCR were as follows: 1 \times (3 min 98°C); 35 \times (1 min 95°C; 2 min 58°C, 1 min 72°C) and 1 \times (7 min 72°C). The PCR reaction was performed using a Mastercycler Gradient (Eppendorf, Hamburg, Germany).

Mutagenesis Methods

Random Mutagenesis (epPCR)

Random mutagenesis of the whole *B. subtilis* lipase gene was performed by error-prone polymerase chain reaction (epPCR) (Zhou *et al.*, 1991). The upper 30 bp primer mut1up 5'-CTCCTC-GCTGCCAGCCGGCGATGGCCATG-3' and the lower 29 bp primer mut1low 5'-GCCGCAAGCTT-GTCGACGAGCTCTCATTA-3' were used in a standard PCR reaction to introduce unique *MlsI* and *HindIII* restriction sites for direct cloning into the expression vector pET22b. An error rate of 2–4 base substitutions per gene was achieved by using the following reaction mix: 5 pmol of each primer, 75 mM Tris/HCl buffer (pH 8.8), 20 mM $(NH_4)_2SO_4$, 6.0 mM $MgCl_2$, 0.15 mM $MnCl_2$, 0.2 mM dNTPs, 0.1% Tween 20, 1 ng template DNA (pET22lipA plasmid) and 2.5 U *Taq*-polymerase (Eurogentec, Seraing, Belgium). For epPCR with lower or higher error rates, the concentration of $MnCl_2$ was varied as described elsewhere (Jaeger *et al.*, 2001).

Single- and Multiple-site Saturation Mutagenesis

Saturation mutagenesis was performed using a megaprimer PCR mutagenesis method as described by Baretino *et al.* (1994). In the first PCR reaction, the megaprimer harboring the desired point mutation(s) was amplified by using a mutagenesis primer

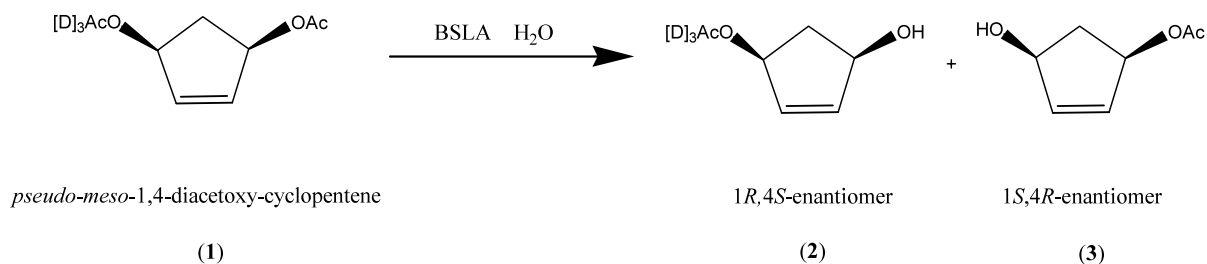


FIGURE 1 The asymmetric hydrolysis of the model compound *meso*-1,4 diacetoxy-2-cyclopentene was determined by using a high-throughput ESI-MS system. The substrate (*pseudo-meso*-1,4-diacetoxy-2-cyclopentene) was deuterium labeled to follow the formation of chiral alcohols (2) and (3) by differences in their mass spectrum.

carrying a randomized codon at the desired position and the upstream primer mut1up. In the second PCR reaction, the full-length gene was amplified using the megaprimer together with mut1up and an additional downstream primer mutSlow 5'-ATA-TAAGCTTCAGCAAACAGCTATGACCATGATATCGAATTC-3'.

For multiple-site saturation mutagenesis (MSSM), the megaprimer method was used with mutagenesis primers simultaneously saturating three amino acid positions: MSSM1 5'-AATCAGGCTGTTGACSNNGCTGCTGTASNNAAGGCCSNNGTGTCCAACGC-CATG-3' and MSSM2 5'-TTCATCTAAAACCTT-SNNCACSNNTCGTGATAATACCGGTCC ATTSN-NATAATTTGTGCCTGT-3' (mutated codons are underlined). MSSM1 introduced mutations at amino acid positions 50, 58 and 60; and MSSM2 at amino acid positions 157, 160 and 164.

Expression and Screening

The PCR-amplified *lipA* mutant genes were cloned into vector pET22b (Novagen, Madison, USA) and the resulting plasmids were transformed into *E. coli* BL21(DE3) (Novagen, Madison, USA). The clones were cultured at 37°C in 96 deep well microtiter plates filled with 1 ml of LB/M9 medium (10 g/l tryptone, 5 g/l yeast extract, 5.5 g/l NaCl, 4 g/l glucose, 0.25 g/l MgSO₄ × 7H₂O, 0.02 g/l CaCl₂, 7 g/l Na₂HPO₄ × 2H₂O, 3 g/l KH₂PO₄, 1 g/l NH₄Cl) supplemented with 100 µg/ml carbenicillin. After 6 hours of shaking at 37°C (OD₅₈₀ = 0.5–0.7) lipase expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.3 mM. The induced culture was grown at 37°C and the cells were separated from the culture by centrifugation at 5000 g for 10 min. An aliquot of 100 µl from the culture supernatant was taken from each well and pipetted into another 96 deep well microtiter plate containing 800 µl of 10 mM Na₂HPO₄/KH₂PO₄ buffer (pH 7.5) and 100 µl of the substrate dissolved in dimethylsulfoxide (100 mM). After 24 h of shaking at room temperature, the reaction solution was extracted with ethyl acetate and screened by electrospray ionization mass spectroscopy (ESI-MS) (Reetz *et al.*, 1999). Variants showing an improved enantioselectivity were reanalyzed independently by chiral gas chromatography. The corresponding mutant genes were sequenced using standard T7-promoter and T7-terminator primers (Medigenomix, Martinsried, Germany).

Stability of Wild Type and Variant Lipases

The thermal stability of the enzymes was determined after expression of the wild type protein and different variants in *E. coli* BL21(DE3) by incubation

of the enzyme-containing supernatants for 2 h at 45°C. The residual enzymatic activities were determined spectrophotometrically using *p*-nitrophenyl-caprylate as the substrate (Eggert *et al.*, 2000).

RESULTS AND DISCUSSION

Directed Evolution of BSLA using Iterative Cycles of epPCR

Directed evolution was used to improve the enantioselectivity of BSLA in the asymmetric hydrolysis of *meso*-1,4-diacetoxy-2-cyclopentene with the formation of chiral alcohols (Fig. 1). This reaction does not constitute a kinetic resolution and can thus be carried out to 100% conversion. Screening was done by electrospray ionisation mass spectrometry (ESI-MS) using the deuterium labeled *pseudo-meso* substrate. The wild-type enzyme leads to an *ee*-value of only 38% in favor of the (1*R*, 4*S*) enantiomer (Fig. 1).

The initial strategy was based on random mutagenesis of the lipase gene using epPCR with a low mutagenesis frequency resulting in one amino acid exchange per variant and subsequent high throughput screening based on ESI-MS. In the first generation created by low error rate epPCR, about five improved mutants were identified, the best one resulting in an *ee*-value of 48% in the test reaction. In parallel, the screening of a first generation prepared by high error rate epPCR corresponding to 2–3 amino acid exchanges per variant resulted in five variants with *ee*-values up to 58% *ee* (Table I). These variants parented a second generation of mutants created by epPCR with high error rate. The total number of second-generation libraries contained 15000 variants. Four enantioselective lipase variants with enantioselectivities up to *ee*-values of 69% were identified (Table I).

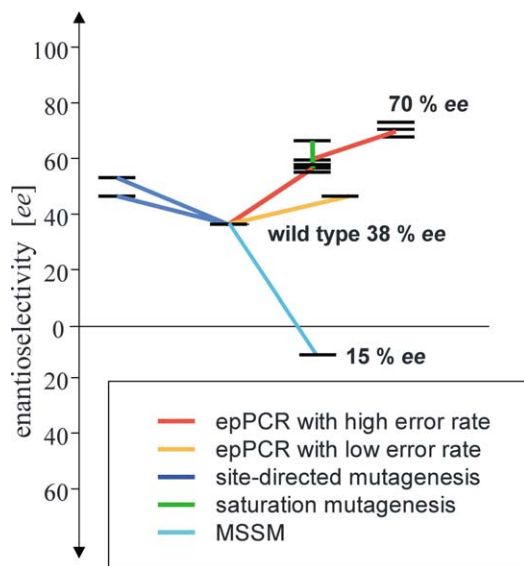
The best variants of the second generation libraries were used for a third round of epPCR. Although 9000 variants were screened, variants with a further improved enantioselectivity could not be identified (Table II).

The DNA sequence analysis of the lipase variants listed in Table I allowed us to identify 'hot-spot positions' important for the enantioselectivity of the enzyme. Further enzyme optimization was achieved by saturation mutagenesis at the following amino acid positions: Ile22, Tyr49, Asn50, Gln60, Leu124 and Gln164. Additionally, multiple site saturation mutagenesis (MSSM) was performed at two regions previously identified as being important for enantioselectivity comprising amino acid positions Asn50, Phe58 and Gln60 (MSSM1) and Ile157, Leu160 and Gln164 (MSSM2). This round of mutagenesis and screening lead to the identification of a novel BSLA variant with inverted enantioselectivity

TABLE I Enzyme variants with improved enantioselectivity towards the model substrate

Generation (mutagenesis method)	Variant	<i>ee</i> [%]	Amino acid exchange
1 st (low error rate epPCR)	wild-type	38	–
	–	40–50	n.d.
1 st (high error rate epPCR)	G1-II-2H12	58	I22V Q164H
	G1-II-3-H6	57	I22T L114P
	G1-II-2-A7	54	L124S
	G1-III-10-C10	56	N50S
	G1-III-13-G9	54	Q60R
2 nd (high error rate epPCR)	G2-II-1-C12	64	I22V L160Q Q164H
	G2-II-9-E1	69	I22T F58L L114P
	G2-II-5-E4	65	L124S I157N
	G2-II-5-E10	64	M78T L124S S130G
(saturation mutagenesis)	Q60N	65	Q60N
	Q60L	64	Q60L
(multiple site saturation mutagenesis)	MSSM1	38–54	n.d.
	MSSM2	15 rev.	n.d.

n.d. = not determined.

FIGURE 2 Directed evolution of enantioselective *B. subtilis* lipase. The mutagenesis methods used to evolve enantioselective variants are indicated by different colors.

(15% *ee*, rev.). The results of this directed evolution approach are summarized in Fig. 2.

Biochemical Characterization of Enantioselective Lipase Variants

In total, 14 enantioselective BSLA variants in the asymmetric hydrolysis of the model substrate were identified (Table I). However, after a third round of epPCR mutagenesis, improved variants were not obtained although about 9000 variants were screened. This result suggested that the improvement of BSLA enantioselectivity was either impossible or was caused by otherwise deleterious mutations. Therefore, we decided to compare enzyme activities and stabilities of the variants to those of the wild-type enzyme.

The specific activities of improved BSLA variants purified by phenyl sepharose chromatography (Egert *et al.*, 2000) were different from the wild-type enzyme. However, all improved variants still

TABLE II Distribution of active variants in the different epPCR generations

Generation	epPCR method	Active variants [%] ¹	Screened variants ²	Max. <i>ee</i> [%] ³
1 st	low error rate	85	1000	50
1 st	high error rate	60	4000	58
2 nd	high error rate	45	5 × 3000	69
3 rd	high error rate	10	3 × 3000	65–69

¹percentage of lipolytically active BSLA variants tested in a pre-screening on tributyrin agar plates.

²number of active enzyme variants screened for enantioselectivity in the asymmetric hydrolysis of *meso*-1,4-diacetoxy-2-cyclopentene.

³maximum *ee*-values identified from screening.

showed enzymatic activity towards the standard lipase substrate *p*-nitrophenyl caprylate indicating that lipolytic activity itself was retained also after the third round of random mutagenesis.

Additionally, the protein stability of selected variants and the wild-type lipase was compared by determination of their residual lipolytic activity after incubation for two hours at 45°C. The wild-type lipase was stable under these conditions, but most variants showed a decrease in lipolytic activity over time, indicating a decreased stability at elevated temperatures as compared to the wild-type enzyme. A plot of the enantioselectivities of the lipase variants versus their respective (thermo)stabilities revealed a negative correlation: the higher the enantioselectivities of the enzymes the lower were their stabilities (Fig. 3). Variants G2-II-1C12, G2-II-9E1 and G2-II-5E10 (see Table I) each containing three amino acid substitutions were selected to parent the third generation. Obviously, the mutations present in these variants not only lead to an increased enantioselectivity but also negatively affected their stability.

These results suggest that, in contrast to larger proteins of $M_r \geq 30$ kDa, small enzymes like BSLA (181 amino acids, M_r 19.4 kDa) are generally more susceptible to amino acid substitutions exerting a negative effect on stability and activity. Enzymes of smaller size have naturally been evolved as compact structures as suggested by the crystal structure of BSLA (van Pouderoyen *et al.*, 2001). A larger number of amino acid substitutions therefore dramatically increase the chance of causing negative effects on their overall structure. As a consequence, directed evolution of such enzymes should start with the

preparation of a high diversity first generation library carrying a low number of mutations (1–2 amino acid exchanges per protein molecule). Accordingly, we observed that saturation mutagenesis seemed to have a better potential to improve the enzyme's enantioselectivity against the model substrate while maintaining its stability and activity as seen for variants I22V, Q60L, Q60R, Y49C and N50S (bold black letters in Fig. 3).

Directed Evolution of BSLA using Complete Saturation Mutagenesis

Single base mutations as introduced by epPCR cannot result in the creation of all theoretically possible amino acid substitutions. The construction of a mutant library containing all possible single amino acid exchanges would be necessary to achieve this ambitious goal. We have saturated every single amino acid position in BSLA by separate saturation mutagenesis of the codons encoding amino acids 1–181 of the native enzyme. The mutant genes were cloned and the enzymes were overexpressed in *E. coli*. In this ongoing project, several different variants with improved (*ee*-value of 65% in favor of 1*S*,4*R*) and inverted (*ee*-value of 56% in favor of 1*R*,4*S*) enantioselectivities towards the model substrate (Fig. 1) were identified. Several BSLA variants with improved specific activities were identified as well. Interestingly, most of these variants proved to be stable, presumably due to the low number of amino acid exchanges per protein molecule (Funke *et al.*, 2003).

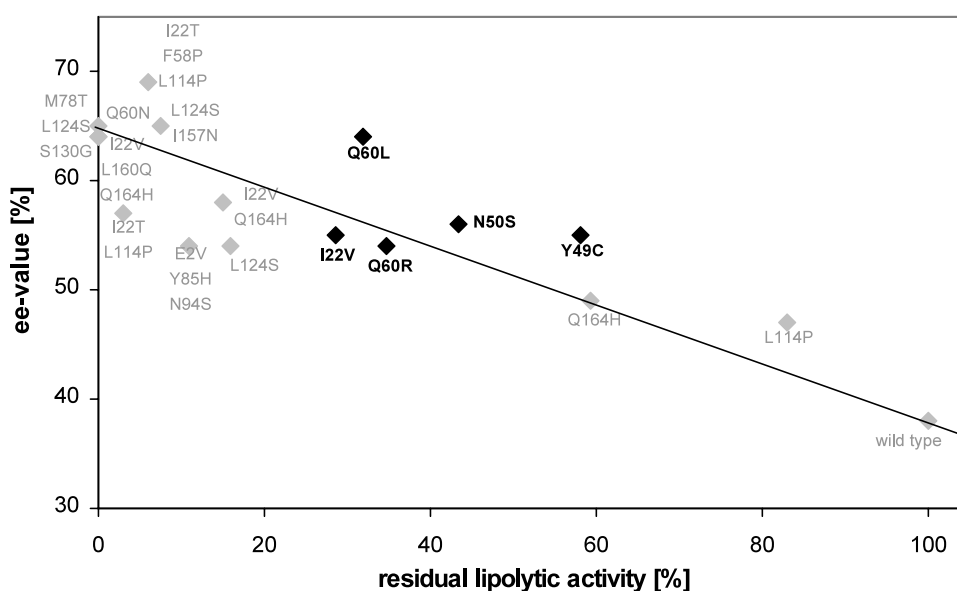


FIGURE 3 Thermostability of wild-type and variant lipases. The residual lipolytic activities were determined after 2 h of incubation at 45°C. The wild-type LipA protein was (thermo)stable under these conditions and its activity was defined as 100%. BSLA-variants showing improved enantioselectivity and maintaining their stability and activity are highlighted in bold black letters.

CONCLUSIONS AND FUTURE PROSPECTS

Directed evolution is a successful strategy to improve enzyme properties such as specific activities, substrate specificities or stabilities and optimize biocatalytic processes (Petrounia and Arnold, 2000; Zhao *et al.*, 2002). Additionally, it is a powerful tool to create enantioselective biocatalysts (Reetz *et al.*, 1997; Jaeger *et al.*, 1999; Liebeton *et al.*, 2000; Jaeger *et al.*, 2001; Jaeger and Eggert, 2002; Reetz and Jaeger, 2002). Success depends on the effective combination of different mutagenesis methods with efficient screening or selection procedures. The quality of the first generation library is of key importance because its variants usually parent all subsequent generations.

Although directed evolution works even without any knowledge of an enzyme's structure or reaction mechanism (Petrounia and Arnold, 2000), a given project may nevertheless turn out to be time-consuming and cumbersome. According to our experience, the knowledge of a three-dimensional protein structure can significantly speed up a directed evolution approach because it may allow the size of the sequence space to be narrowed down. Despite its value in identifying the spacial positions of important amino acid residues, a crystal structure itself does not reflect the time-resolved catalytic reaction mechanism. Therefore, we have recently started to simulate the BSLA-catalyzed substrate hydrolysis by using combined quantum mechanical and molecular mechanical calculations (QM/MM) (Fig. 4). These computer calculations describe a dynamic reaction by treating the active site residues quantum mechanically, while the surrounding en-

vironment is simulated by a force field method (Schöneboom *et al.*, 2002). BSLA is an ideal candidate enzyme for QM/MM calculations because it is a small enzyme with a known crystal structure (van Pouderoyen *et al.*, 2001).

Hopefully, this approach will result in a more detailed understanding of the enzyme's topology and its changes in the course of the enantioselective reaction. In the end, a structure and theory assisted analysis of a directed evolution approach will facilitate the understanding of an enantioselective enzyme reaction and, at the same time, help to accelerate the creation of enantioselective lipases useful for industrial applications.

References

- Baretino, D., Feigenbutz, M., Valcárcel, R. and Stunnenberg, H.G. (1994) "Improved method for PCR-mediated site-directed mutagenesis", *Nucleic Acids Res.* **22**, 541–542.
- Bornscheuer, U.T. and Pohl, M. (2001) "Improved biocatalysts by directed evolution and rational protein design", *Curr. Opin. Chem. Biol.* **5**, 137–143.
- Eggert, T. and Jaeger, K.-E. (2003) "Directed evolution by random mutagenesis: a critical evaluation", In: Svendsen, A., ed, *Enzyme functionality: Design, Engineering and Screening* (Marcel Dekker, New York) (in press).
- Eggert, T., Pencreac'h, G., Douchet, I., Verger, R. and Jaeger, K.-E. (2000) "A novel extracellular esterase from *Bacillus subtilis* and its conversion to a monoacylglycerol hydrolase", *Eur. J. Biochem.* **267**, 6459–6469.
- Funke, S.A., Eipper, A., Eggert, T., Jaeger, K.-E., Reetz, M.T. (submitted for publication).
- Jaeger, K.-E. and Eggert, T. (2002) "Lipases for biotechnology", *Curr. Opin. Biotechnol.* **13**, 390–397.
- Jaeger, K.-E., Dijkstra, B.W. and Reetz, M.T. (1999) "Bacterial biocatalysts: Molecular biology, three-dimensional structures, and biotechnological applications of lipases", *Annu. Rev. Microbiol.* **53**, 315–351.
- Jaeger, K.-E., Eggert, T., Eipper, A. and Reetz, M.T. (2001) "Directed evolution and the creation of enantioselective biocatalysts", *Appl. Microbiol. Biotechnol.* **55**, 519–530.
- Liebeton, K., Zonta, A., Schimossek, K., Nardini, M., Lang, D., Dijkstra, B.W., Reetz, M.T. and Jaeger, K.-E. (2000) "Directed evolution of an enantioselective lipase", *Chem. Biol.* **7**, 209–218.
- Lutz, S., Ostermeier, M., Moore, G.I., Maranas, C.D. and Benkovic, S.J. (2001) "Creating multiple-crossover libraries independent of sequence identity", *Proc. Natl. Acad. Sci.* **98**, 11248–11253.
- Petrounia, I.P. and Arnold, F. (2000) "Designed evolution of enzymatic properties", *Curr. Opin. Biotechnol.* **11**, 325–330.
- Reetz, M.T. (2001) "Combinatorial and evolution-based methods in the creation of enantioselective catalysts", *Angew. Chem. Int. Ed.* **40**, 284–310.
- Reetz, M.T. (2002) "Lipases as practical biocatalysts", *Curr. Opin. Chem. Biol.* **6**, 145–150.
- Reetz, M.T. and Jaeger, K.-E. (2002) "Directed evolution as a means to create enantioselective enzymes for use in organic chemistry", In: Brakman, S. and Johnsson, K., eds, *Directed Molecular Evolution of Proteins or How to Improve Enzymes for Biocatalysis* (WILEY-VCH, Weinheim, Germany), pp 245–279.
- Reetz, M.T., Zonta, A., Schimossek, K., Liebeton, K. and Jaeger, K.-E. (1997) "Creation of enantioselective biocatalysts for organic chemistry by *in vitro* evolution", *Angew. Chem. Int. Ed.* **36**, 2830–2832.
- Reetz, M.T., Becker, M.H., Klein, H.W. and Stöckigt, D. (1999) "A method for high-throughput screening of enantioselective catalysts", *Angew. Chem. Int. Ed.* **38**, 1758–1761.

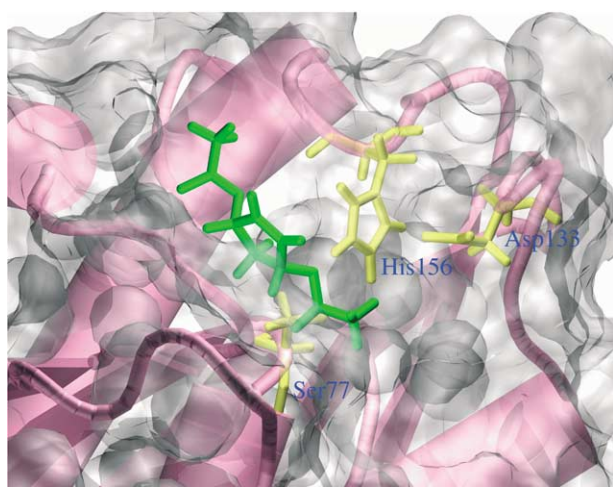


FIGURE 4 QM/MM simulation of *meso*-1,4-diacetoxy-2-cyclopentene hydrolysis catalyzed by BSLA calculated on the basis of the crystal structure. Quantum mechanical calculations were applied for the substrate (green) and the side chains of the catalytic triad residues Ser77, Asp133, and His156 (yellow). The remaining part of the enzyme was treated by a force field method. For clarity, the 20 Å water sphere capping the active site is not displayed.

- Rosenau, F. and Jaeger, K.-E. (2003) "Overexpression of biocatalysts in *Pseudomonas*", In: Svendsen, A., ed, *Enzyme functionality: Design, Engineering and Screening* (Marcel Dekker, New York) (in press).
- Rouhi, A.M. (2002) "Chiral roundup", *Chem. Eng. News* **80**(23), 43–50.
- Shao, Z., Zhao, H., Giver, L. and Arnold, F.H. (2000) "Random-priming *in vitro* recombination: an effective tool for directed evolution", *Nucleic Acids Res.* **26**, 681–683.
- Schöneboom, J.C., Lin, H., Reuter, N., Thiel, W., Cohen, S., Ogliaro, F. and Shaik, S. (2002) "The elusive oxidant species of cytochrome p450 enzymes: characterization by combined quantum mechanical/ molecular mechanical (QM/MM) calculations", *J. Am. Chem. Soc.* **124**, 8142–8151.
- Stemmer, W.P.C. (1994) "Rapid evolution of a protein *in vitro* by DNA shuffling", *Nature* **370**, 389–391.
- Stemmer, W.P.C. (1994) "DNA shuffling by random fragmentation and reassembly: *In vitro* recombination for molecular evolution", *Proc. Natl. Acad. Sci.* **91**, 10747–10751.
- Stinson, S.C. (2001) "Chiral pharmaceuticals", *Chem. Eng. News* **79**(40), 79–97.
- van Pouderoyen, G., Eggert, T., Jaeger, K.-E. and Dijkstra, B.W. (2001) "The crystal structure of *Bacillus subtilis* lipase: a minimal α/β -hydrolase fold enzyme", *J. Mol. Biol.* **309**, 215–226.
- Zhao, H., Giver, L., Shao, Z., Affholter, J.A. and Arnold, F. (1998) "Molecular evolution by staggered extension process (StEP) *in vitro* recombination", *Nat. Biotechnol.* **16**, 258–261.
- Zhao, H., Chockalingam, K. and Chen, Z. (2002) "Directed evolution of enzymes and pathways for industrial biocatalysis", *Curr. Opin. Biotechnol.* **13**, 104–110.
- Zhou, Y.H., Zhang, X.P. and Ebright, R.H. (1991) "Random mutagenesis of gene-sized DNA molecules by use of PCR with *Taq* DNA polymerase", *Nucleic Acids Res.* **19**, 6052.

